PRO EXPERIMENTIS

The Use of Airtight Culture Flasks in Anaerobic Studies

The requirement for oxygen by various cell strains cultivated *in vitro* has not yet been systematically investigated, and many studies suffer from the use of culture vessels which are not strictly airtight. This criticism applies especially to reports on anaerobic cultures, where the results are conflicting. For example, HARRIS[1] states that young rat fibroblasts survive and grow equally well in the absence of oxygen as in air, although with his electrochemical method of measuring the oxygen tension an unaccountable residual current consistently occurred in 'oxygen-free' conditions, and the culture containers were sealed only with liquid paraffin, suggesting the possibility of a steady leak. DALES[2] and BROSEMER and RUTTER[3] report that mammalian fibroblasts do grow anaerobically, but at reduced rates, although again the degree of anaerobiosis in each case may be questioned. DALES used silicone stoppers with pieces of glass tubing for gas inlet and outlet, but did not say how these were sealed after gassing, if at all. BROSEMER and RUTTER do not describe their 'specially designed culture vessels' for control of the gaseous environment, so no judgment is possible. GIFFORD[4], who reports varying degrees of survival of HeLa cells anaerobically according to the culture medium employed, used the retention of positive pressure in flasks sealed with rubber diaphragms as an indication of airtightness. As will be shown below, this is not necessarily a valid method.

Strictly anaerobic conditions can be obtained either by continuously flushing cultures with an absolutely oxygen-free atmosphere, or by gassing flasks with a nearly oxygen-free atmosphere and sealing them completely, allowing the cells to use up any residual oxygen. Because of the difficulty of knowing whether one has an absolutely oxygen-free gas, anaerobic studies are probably best conducted in truly airtight flasks.

In order to test the airtightness of flasks, use was made of FILDES and MCINTOSH's anaerobic test solution[5], made up of equal parts of $6\times 10^{-3}M$ NaOH, 0.015% aqueous methylene blue and 6% dextrose. When heated, the methylene blue is reduced to the colorless leuco form by glucose; the reaction was found to be negligible at room temperature, about 3 μmoles/l/day in oxygen-free conditions. On the admission of oxygen, the blue, oxidized form of the dye reappears.

It was found empirically that at a depth equal to the thickness of a T_{15} culture flask[6], the limit of visual detection of oxidized methylene blue was between 1.56×10^{-5} and $1.56\times 10^{-6}M$. If 2 ml of test solution are placed in a 15 ml culture flask and decolorized, then the return of a detectable blue color ($1.56\times 10^{-5}M$ methylene blue) is indicative of a minimum oxygen tension of approximately 0.0025% at 20°C and 760 mm Hg pressure, or about 0.4 μl O_2. The time required for such recolorization gives an estimate of the rate of oxygen entry into the system, and of its significance to 'anaerobic' studies. For example, 10^6 strain L mouse cells utilize around 5 μl O_2/h in air[7], and a 15 ml flask in which detectable blue appeared in 5 min could therefore supply this number of cells with nearly enough oxygen to respire at a maximum level. (Whether they would in fact respire maximally at such a low oxygen tension is another question, which may well depend on the strain of cells being investigated.)

Because it is often assumed that when a rubber or silicone stopper is used a flask is airtight, especially if it is coated with vaseline-paraffin (vaspar) mixtures, this point was tested. A T_{15} flask containing decolorized test solution was closed with a silicone rubber stopper containing two fine glass tubes, one for a gas inlet and one for the outlet. After gassing with oxygen-free nitrogen, the flask was sealed by melting the tips of the glass tubing. In a few minutes the methylene blue began to recolorize. The same result was always obtained, even if the stopper was coated with vaspar.

It appeared possible that the pores of the stoppers themselves contained sufficient oxygen to reoxidize the methylene blue. This was tested by constructing a large Y-tube, two ends of which were closed. Into one arm pieces of stopper were placed, and into the other, an aliquot of decolorized anaerobic test solution. The tube was gassed with nitrogen, then sealed by fusing the open arm in a flame. In a short time the stoppers had released enough oxygen to partially recolorize the methylene blue. In Y-tubes containing only test solution no recolorization occurred. Therefore, silicone stoppers contain a demonstrable amount of oxygen in their pores, which is released into the flasks. Although published data on the permeability to oxygen of silicone stoppers are not available, the permeability to air of various vulcanized rubbers is of the order of 10^{-10} cm³/cm²/cm thickness/cm Hg/sec, or, for a pressure difference of 15 cm Hg, 0.005 μl/h of oxygen through a unit cube. This is two to three orders of magnitude less than the rate found here, and suggests that oxygen in the pores of the stopper and leaks around the edges of the stopper and around the pieces of glass tubing were the major factors in oxygen entry.

Maintaining a positive pressure inside a flask sealed with a rubber membrane, as used by GIFFORD[4], does not demonstrate the absence of oxygen entry into the flask unless it can be shown that *no* decrease in the internal pressure has taken place during the experimental period. If we assume that the membrane is chemically inert and behaves similarly towards oxygen and nitrogen, a consideration of the laws of partial pressure and the kinetics of gas movement into and out of a flask which contains 98% N_2 at 1.026 atmospheres, as used by GIFFORD[4], shows that for every 1.23 molecules of nitrogen leaving the flask, 1 molecule of oxygen enters. (GRAHAM's law of effusion[8] can be used to calculate the rates, r_{in} and r_{out}, for the movement of both nitrogen and oxygen at the pressures stated. The ratio of $(r_{out}-r_{in})_{N_2}/(r_{in}-r_{out})_{O_2}$ then gives the net movement of nitrogen out of the flask per unit oxygen entering.) In a flask of 30 ml volume, containing 3×10^6 cells (as used by GIFFORD) respiring at around 15 μl/h, an inward diffusion of oxygen sufficient to supply these cells for one day (300 μl O_2) would occur simultaneously with a loss of 369 μl nitrogen, and a drop in positive pressure from the original level of 1.026 atmospheres to one of 1.014 atmospheres, assuming that the cells use up all the oxygen and no gaseous CO_2 is produced by them. It should also be noted that $r_{out_{N_2}}$ is

[1] H. HARRIS, Brit. J. exp. Path. *37*, 512 (1956).
[2] S. DALES, Can. J. Biochem. Physiol. *38*, 871 (1960).
[3] R. W. BROSEMER and W. J. RUTTER, Exp. Cell Res. *25*, 101 (1961).
[4] G. E. GIFFORD, Exp. Cell Res. *31*, 113 (1963).
[5] P. FILDES and J. MCINTOSH, Brit. J. exp. Path. *2*, 153 (1921).
[6] W. R. EARLE and F. HIGHHOUSE, J. nat. Cancer Inst. *14*, 841 (1954).
[7] B. S. DANES and J. KIELER, C. R. Trav. Lab. Carlsberg, Ser. chim. *31*, 61 (1958).
[8] T. GRAHAM, Philos. Trans. *136*, 573 (1846).

based on the initial nitrogen pressure, and the rate of net loss of nitrogen will decrease as the internal pressure drops, whereas, assuming the oxygen is utilized by the cells, its rate of entry will remain unchanged.

In order to overcome the possibility of uncontrolled leakage of oxygen into culture flasks, T_{15} flasks were modified as shown in the Figure, the ground joints being lubricated with high-vacuum silicone grease. When such flasks were tested by the method described above, no recolorization of the methylene blue was observed, even after eight days, although as soon as the flasks were opened to air the color returned. Less than 2 µl of O_2 therefore had entered such a flask during this time, even after allowance for the reverse (reduction) reaction. A rubber band fastened to the 'rabbit ears' insured a tight seal when the flasks were used at a higher temperature (37°C) in the incubator.

Because we know so little about the respiration and growth of cells cultured at reduced oxygen tensions, it is clear that statements regarding the ability of cells to survive and grow in the absence of oxygen are not valid unless it can be shown that truly anaerobic conditions have been maintained. It is quite possible that there exists a minimum oxygen tension, at which cells may respire submaximally, where growth is not inhibited, and that below this point growth is progressively slowed and eventually stopped, whereupon cell death ensues. In a further paper evidence in support of this view will be presented for cultures of adult mouse fibroblasts[9].

Zusammenfassung. Eine kritische Untersuchung verschiedener Methoden für anaerobe Gewebskultur zeigte, dass in den meisten Einrichtungen die Anwesenheit von Sauerstoff nicht ausgeschlossen werden kann. Eine Modifikation des standardisierten Kulturgefässes wurde entwickelt, die das Halten der Kulturen unter völligem Luftabschluss möglich macht.

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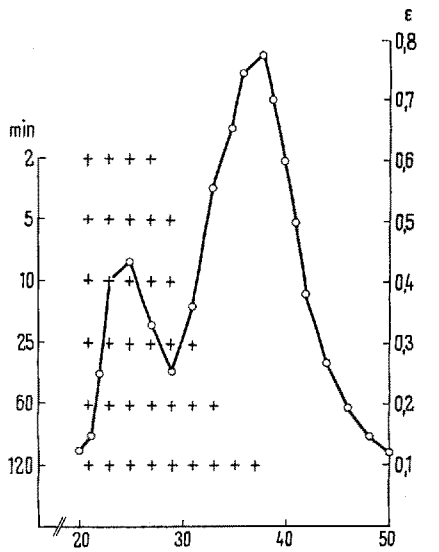
Serienbestimmung der Phospholipase-A-Aktivität in chromatographischen Fraktionen

Zur Auswertung chromatographischer Trennungen von tierischen Giften entwickelten wir ein einfaches, für Serienbestimmungen geeignetes Verfahren zum Nachweis von Phospholipase A. In unserer Methode machten wir uns die Entdeckung von HANAHAN[1] zunutze, dass Phospholipase A auch in ätherischer Lösung aus Lecithin eine Fettsäure abspaltet. Das entstehende Lysolecithin ist in Äther schwer löslich und fällt aus.

Von den zu untersuchenden Fraktionen werden 0,01–0,02 ml in Reagenzgläser pipettiert und 1 ml einer ätherischen Lecithinlösung zugefügt. Nach Durchmischen wird die Serie der Röhrchen in bestimmten Zeitabständen beobachtet und verzeichnet, welche Röhrchen eine Trübung durch ausgefallenes Lysolecithin zeigen. Markiert man die positiven Röhrchen auf einem Diagramm mit einem Kreuz und kreuzt man bei jeder neuen Ablesung auch die schon vorher positiv gewesenen Röhrchen wieder an, so erhält man eine Darstellung, die das Maximum und die Verteilung des Fermentes in der Fraktionsserie erkennen lässt (Figur).

Die Lecithinlösung besteht aus Äther (100 vol), Picolin (10 vol) und 4,5 mmolar wässriger Lösung von $CaCl_2$ (1 vol). In 100 ml dieser Mischung werden 1 g gereinigtes Lecithin gelöst. Zur Reinigung wurde Ovo-Lecithin (Merck) in Chloroform-Methanol (1 : 1) gelöst und an Al_2O_3 (Woelm, neutral) chromatographiert[2]. Bei kühler und dunkler Aufbewahrung ist die Lösung praktisch unbegrenzt brauchbar. Schwierigkeiten können dann auftreten, wenn für die Chromatographie der Phospholipase



Abtrennung von Phospholipase A aus einem vorgereinigten Bienengift an Sephadex G 75 (50 × 1,5 cm Säule) in Wasser. Abszisse: Zahl der Fraktionen à 2 ml. Ausgezogene Linie: Proteinmenge nach Folin-Lowry[3]. Kreuze: Positiver Ausfall des Phospholipase-A-Tests. Bei der Ablesung nach 2 min waren positiv Röhrchen 21–27, nach 5 min auch Rohr 29 und schliesslich nach 2 h alle weiteren Röhrchen bis Nr. 37.

[1] D. J. HANAHAN, J. biol. Chem. *195*, 199 (1952).

[2] D. N. RHODES und C. H. LEA, Biochem. J. *65*, 526 (1957).

[3] H. STEGEMANN, HOPPE-SEYLERS Z. *319*, 82 (1960).